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MOLECULAR MECHANISMS OF CARDIOVASCULAR CALCIFICATION

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Molecular Mechanisms of Cardiovascular Calcification

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ABSTRACT

Cardiovascular calcification is a pathophysiological process characterized by the deposition of calcium-phosphate crystals in the arteries and the leaflets of the heart valves. In the arteries calcification causes arterial stiffness, which may lead to poor cardiac perfusion, systolic hypertension and heart failure. In the aortic valve, calcification causes left ventricular outflow obstruction. Currently, no medical treatment exists to halt or reverse cardiovascular calcification. For that reason, understanding the molecular mechanisms underlying cardiovascular calcification is of particular importance.

Molecularly, cardiovascular calcification is a continuum comprising intertwined physicochemical and biologically active processes. In particular, cardiovascular calcification commences when cells become overburdened by the mineral imbalance typical of chronic kidney disease (CKD), or the unresolved inflammation characteristic of atherosclerosis and aortic valve stenosis (AVS). These alterations in homeostasis lead to changes in the fate and phenotype of structural cells such as vascular smooth muscle cells (VSMCs) and valvular interstitial cells (VICs). This phenotypic switch is characterized by: the loss of calcification inhibitors, an increase in pro-osteogenic signaling, changes in proliferation, abnormal processing and synthesis of extracellular matrix (ECM), and alterations in autophagy.

In the current thesis, three pathways relevant to cardiovascular calcification are discussed. First, in **Articles I** and **II**, the G-protein coupled receptor ChemR23 arises as a promoter of a synthetic and proliferative VSMC phenotype, prone to phosphate-induced calcification. Importantly, this phenotype could be reverted by genetic deletion of ChemR23, and calcification was inhibited by the ChemR23 ligands: RvE1 and chemerin. Translationally, chemerin was negatively associated with coronary artery calcification in CKD patients. Moreover, in **Article III**, ChemR23 expressed in macrophages, promoted the resolution of inflammation, and inhibited VSMC proliferation in a mouse model of intimal hyperplasia. Secondly, **Article IV** demonstrates that iron, preferentially present in the calcified regions of the aortic valve, accumulated in VICs. This uptake of iron enhanced VIC proliferation and actively contributed to the ECM remodeling. Finally, **Article V** reveals a detrimental role of the second generation tyrosine kinase inhibitor nilotinib on the aortic valve. *In vivo*, nilotinib promoted aortic valve thickening. *In vitro*, nilotinib enhanced VIC osteoblastic trans-differentiation, increased calcification and inhibited autophagy. Mechanistically, nilotinib preferentially inhibited the most abundant collagen sensing tyrosine kinase in the valve: the discoidin domain receptor 2.

Overall the results from this thesis suggests that changes in VSMC and VIC phenotype, as well as alterations in the ECM content and sensing can have profound effects on cardiovascular calcification, and therefore serve as potential therapeutic targets.

LIST OF SCIENTIFIC PAPERS

I. Carracedo, M.; Artiach, G.; Witasp, A.; Claria, J.; Carlstrom, M.; Laguna-Fernandez, A.; Stenvinkel, P.; Bäck, M.,
The G-protein coupled receptor ChemR23 determines smooth muscle cell phenotypic switching to enhance high phosphate-induced vascular calcification.
Cardiovascular Res. 2018; cvy316

II. Carracedo M, Witasp A, Qureshi A, Brismar T, Stenvinkel P, Bäck M.
Chemerin inhibits vascular calcification through ChemR23 and is associated with lower coronary calcium in chronic kidney disease.
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IV. Laguna-Fernandez A, Carracedo M, Jeanson G, Nagy E, Eriksson P, Caligiuri G, Franco-Cereceda A, Bäck M.
Iron alters valvular interstitial cell function and is associated with calcification in aortic stenosis.
Eur Heart J 2016;37(47):3532-3535.

V. Carracedo M, Artiach G, Pouwer M, Persson O, Saliba P, Ehrenborg E, Eriksson P, Princen H, Franco-Cereceda A, Bäck M.
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Upregulated autophagy in calcific aortic valve stenosis confers protection of valvular interstitial cells.
Int J Mol Sci. 2019, 20, (6).

CONTENTS

LIST OF ABBREVIATIONS

MSCs	Mesenchymal stem cells
VSMCs	Vascular smooth muscle cells
ACTA2	Actin
CNN1	Calponin
SM22A	Smooth muscle cell protein 22-alpha
CKD	Chronic kidney disease
BMPs	Bone morphogenic proteins
TNAP	Tissue non-specific alkaline phosphatase
MMPs	Matrix metalloproteinases
AVS	Aortic valve stenosis
VECs	Valvular endothelial cells
VICs	Valvular interstitial cells
Runx2	Runt-related transcription factor 2
MGP	Matrix gla protein
OPG	Osteoprotegerin
Ppi	Pyrophosphate
RANKL	Receptor activator of nuclear factor-kappaB ligand
CPPs	Calciprotein particles
eNPP1	Ectoenzyme nucleotide pyrophosphatase/phosphodiesterase-1
PiT-1 and PiT-2	Type III NaPi cotransporters 1 and 2
ECM	Extracellular matrix
MMP13	Matrix metalloproteinase 13
DDR1 and 2	Discoidin domain receptors 1 and 2
ELN	Elastin
TGF-β	Transformin growth facto beta
BMPR2	Bone morphogenetic protein receptor type 2
IL-10	Interleukin 10
GPCR	G protein coupled receptor
PUFAs	Polyunsaturated fatty acids
EPA	Eicosapentanoic acid

DHA	Docosahexaenoic acid
RvE1	Resolvin E1
CVCs	Calcifying vascular cells
MAPK	p38-mitogen-activated protein kinase
PPAR-γ	Peroxisome proliferator-activated receptor-γ
ChemR23	Chemokine like receptor 1
CT	Computed tomography
CAC	Coronary artery calcification
HU	Housenfield units
CVD	Cardiovascular disease
PET	Cositron emission tomography
18F-FDG	18F-fluorodeoxyglucose
18F-NaF	18F-sodium fluoride
FGF23	Fibroblast growth factor 23
oxLDL	Oxidized low density lipoprotein
TNF-α	Tumor necrosis factor alfa
FPN1	Ferroportin
DMT1	Divalent metal transporter 1
RBC	Red blood cell
TKIs	Tyrosine kinase inhibitors
CML	Chronic myeloid leukemia
TKs	Tyrosine kinases
COL1A1	Collagen type 1
$ApoE^{-/-}$	ApoE-deficient mice
$ChemR23^{+/+}$	ChemR23 wild-type mice
$ChemR23^{-/-}$	ChemR23 knock-out mice

1 INTRODUCTION

1.1 Cardiovascular Calcification

Cardiovascular calcification results from the nucleation of calcium and phosphate into calcium-phosphate crystals in the arteries and the leaflets of the heart valves. Cardiovascular calcification is the consequence of three non-mutually exclusive processes: dystrophic calcification, metastatic calcification and heterotopic ossification. Dystrophic calcification is defined as the deposition of calcium and phosphate crystals in soft tissues as a result of injury, disease, and age. On the other hand, in metastatic calcification the deposition of calcium and phosphate occurs in healthy tissue as a consequence of hyperphosphatemia and hypercalcemia [1]. Finally, heterotopic ossification is the organized biomineralization of soft tissue, which exhibits a similar structure as mature bone [2, 3].

1.1.1 Vascular Calcification

Vascular calcification can occur in different locations of the arteries depending on the disease etiology. Arteries are formed by an outermost layer, known as the adventitia; a medial layer, known as the media; and an innermost layer in contact with the blood, known as the intima.

The outermost layer, the adventitia, is composed of fibroblasts, connective tissue, and adipocytes. The adventitia serves as support for the vessels, but also as a niche for mesenchymal stem cells (MSCs). Adventitial MSCs have been implicated in both intimal and medial calcification by serving as a source of osteoblast-like cells [4]. Interestingly, recent reports have shown calcification in the adventitia of ApoE-deficient mice ($ApoE^{-/-}$) fed a high fat diet and autopsy samples of patients who had died of heart failure or acute renal failure [5].

The second layer, the media, is composed of elastin fibers and vascular smooth muscle cells (VSMCs). Under physiological conditions VSMCs are responsible for maintaining a variable contractile tone of the arteries. To achieve this, a wide variety of contractile proteins are expressed, such as actin (ACTA2), calponin (CNN1) and smooth muscle cell protein 22-alpha (SM22A). In addition, VSMCs present a high plasticity, which allows them to undergo diverse phenotypic changes, which will be discussed later in detail.

Medial calcification is most prevalent in diabetes and chronic kidney disease (CKD). CKD refers to the gradual loss of kidney structure and function, which translates in the build-up of phosphate (hyperphosphatemia), calcium (hypercalcemia) and uremic toxins in the blood [6]. Medial calcification causes arterial stiffness, which may lead to poor cardiac perfusion, systolic hypertension and heart failure [7].

The innermost layer, in direct contact with the blood is the intima, which is composed of a monolayer of endothelial cells. This monolayer acts as a selective barrier for cells and molecules, which regulate the vascular tone as well as coagulation and fibrinolysis. In the context of vascular calcification endothelial cells can produce bone morphogenic proteins (BMPs), which are inducers of calcification [8]. Moreover, endothelial cells undergo an

endothelial-mesenchymal transition, thus becoming osteogenic cells [9]. Intimal calcification is characteristic of atherosclerosis. Atherosclerosis is a chronic inflammatory disease characterized by cholesterol accumulation, macrophage infiltration, as well as VSMC migration and proliferation into the intima layer of the arterial wall, known as the atherosclerotic plaque. It is under these conditions, and in this location, where intimal calcification takes place. Calcification of coronary atherosclerotic plaques, in the form of spotty micro calcifications, decrease plaque stability, thus increasing the risk of plaque rupture, thrombus formation, and subsequent myocardial infarction [10 11].

Initially calcification is driven by the presence of inflammatory cytokines and oxidized lipids which promote endothelial activation as well as VSMCs trans-differentiation and apoptosis [12]. One key difference between medial and intimal calcification is that intimal calcification can occur in the absence of hyperphosphatemia due to the strong inflammatory environment [6]. Under inflammation an important source of phosphate comes from the hydrolysis of ATP by the tissue non-specific alkaline phosphatase (TNAP) [13]. Moreover, in intimal calcification, unlike medial calcification, macrophages accumulate. These macrophages, secrete matrix vesicles and matrix metalloproteinases (MMPs) which facilitate the deposition of calcium-phosphate crystals [14].

1.1.2 Valvular Calcification

Calcification of the aortic valve is known as aortic valve sclerosis. When aortic valve calcification causes left ventricular outflow obstruction, it is referred to as aortic valve stenosis (AVS). AVS is a common disease, with a prevalence of 2-7% in populations above 65 years [15]. The incidence and severity of AVS increase with age [16], as well as with several cardiovascular risk factors such as: obesity [17], smoking [18], and renal dysfunction [19]. AVS is a progressive disease with a 1-year mortality of almost 50% in patients with symptomatic AVS [20, 21]. Currently no medical treatment exists to stop the calcification process in the cardiovascular system. With either surgical valve replacement or, transcatheter aortic valve implantation as the only alternatives for AVS patients.

The aortic valve is composed of a monolayer of valvular endothelial cells (VECs), in direct contact with the blood, on both the aortic and ventricular side. These endothelial cells serve as sensors of hemodynamic forces. In between these two layers, three distinct layers of extracellular matrix and valvular interstitial cells (VICs) form the bulk of the valve. Closer to the aortic side is the fibrosa, formed by VICs and collagen. The middle layer is the spongiosa, composed of VICs and glycosaminoglycans. Finally, closest to the ventricular side is the ventricularis, formed of VICs and elastin [22]. VICs are the most abundant cell type in the valve. VIC are phenotypically and functionally diverse. The most abundant population is the pleomorphic fibroblastic VIC, which produce different extracellular matrix (ECM) components and growth factors; and lack myofilaments[23].

At the initial stages of the disease mechanical stress, inflammatory molecules and lipids damage the endothelium, allowing lipids and immune cells to infiltrate, thus promoting

thickening and calcification of the valve. Another mechanism of AVS progression discussed in the current thesis is the extravasation of red blood cells inside the valve called intraleaflet haemorrhage, which occurs as a consequence of endothelial microfissuring [24] and rupture of neovessels [25].

1.2 Molecular Mechanisms of Vascular Calcification

Cardiovascular calcification is a pathophysiological continuum comprising intertwined physicochemical and biologically active processes. The temporality of events leading to cardiovascular calcification remains to be fully elucidated; however, the calcification process can be divided in two intertwined steps: the loss of calcification inhibitors and changes in the phenotype of structural cells.

Initially, cardiovascular calcification commences when cells become overburdened by the mineral imbalance typical of CKD, or the unresolved inflammation characteristic of atherosclerosis and AVS. The excess of calcium, phosphate and uremic toxins, in CKD, and the infiltration of lipids and immune cells in atherosclerosis and AVS promote the proliferative and synthetic phenotype of structural cells [26]. This phenotypic switch is characterized by: the loss of calcification inhibitors both systemically and locally [27-29]; an increase in the pro-osteogenic BMP-2; the subsequent upregulation of TNAP [30]; changes in proliferation; and the abnormal processing of ECM components, such as the degradation of elastin [31, 32]. The combination of these factors is essential for the initial precipitation, nucleation and growth of calcium-phosphate crystals. Especially since the precipitation of calcium and phosphate, under normophosphatemia and the presence of calcification inhibitors, is not thermodynamically favored [33, 34]. Next, these crystals promote the phenotypic switch of VSMCs and VICs into osteoblast like cells. These osteoblasts like cells are responsible for the heterotopic bone formation characteristic of the later stages of vascular calcification, present in 10% to 13% of the calcified valves and arteries [2, 3, 35]. The key molecules overexpressed during this osteoblastic trans-differentiation, and discussed in the current thesis, are the runt-related transcription factor 2 (Runx2) and collagen. The interplay between such processes will be discussed in the following section.

1.2.1 Calcification Inhibitors

Under homeostasis, the deposition of calcium and phosphate is actively inhibited by calcification inhibitors present in the circulation and produced locally by structural cells. Calcification inhibitors can act either by direct physicochemical inhibition of calcium-phosphate crystals, or by inhibiting pro-calcifying molecules and pathways in cells [29]. Some of the most relevant inhibitors of calcification addressed in the present thesis include: matrix gla protein (MGP), osteoprotegerin (OPG), Fetuin-A and pyrophosphate (Ppi).

MGP is a calcification inhibitor produced locally by VSMCs and chondrocytes. To exert its function, the glutamate residues in MGP need to be carboxylated by vitamin-K. When carboxylated, MGP can inhibit calcification by directly binding calcium ions as well as BMP-2 [36], thus inhibiting both the physicochemical and cell mediated calcification [30, 37]. Genetic

deletion of MGP leads to extensive cardiovascular calcification in mice[37]. Interestingly, MGP knock-out mice backcrossed with hypophosphatemic mice (hyp mice) present no vascular calcification, which can be reverted by feeding these mice a high phosphate diet [38].

OPG is a glycoprotein that inhibits osteoclastogenesis by acting as a decoy receptor for the receptor activator of nuclear factor-kappaB ligand (RANKL) [39]. In the vasculature OPG has potent anti-calcifying effects both *in vitro* and *in vivo*, and OPG knock-out mice develop osteoporosis and vascular calcification. In the vasculature the inhibitory mechanisms involve RANKL inhibition, Notch1 inhibition, and cell survival [40, 41].

Fetuin A (or α2-Heremans-Schmid glycoprotein), is a negative acute phase protein produced in the liver and downregulated with inflammation [42], which forms colloidal complexes with calcium and phosphate in plasma [43]. These complexes, referred to as calciprotein particles (CPPs) inhibit growth of calcium-phosphate crystals in the blood and thereby prevent their soft tissue deposition. Interestingly, the development of cardiovascular calcification in the Fetuin A knock-out mice appears to be highly dependent on the strain [44], diet [43] and specially the kidney function [45]. This suggests that fetuin A may prevent cardiovascular calcification only when the calcium phosphate balance is disturbed, whereas its protective effects under physiological conditions may be limited [46].

Ppi is an inorganic molecule derived from the hydrolysis of ATP by the ectoenzyme nucleotide pyrophosphatase/phosphodiesterase-1 (eNPP1), and degraded by TNAP. Ppi inhibits calcification both *in vitro* and *in vivo*, by substituting phosphate thus preventing further mineralization [47]. Importantly, both a lack of production, or increase in degradation of Ppi leads to vascular calcification [48].

1.2.2 Structural Cell Phenotypic Trans-differentiation

The structural cells that comprise both the aortic valve and the arteries, VICs and VSMCs respectively, are quiescent but highly plastic cells. VICs and VSMCs can alter their fate by undergoing complex structural and functional changes. Some of these changes in cell fate which are discussed in the current thesis include: the synthetic and proliferative phenotype, the osteoblastic phenotype, and alterations in autophagy.

1.2.2.1 Synthetic and proliferative phenotype

In response to injury and inflammation, characteristic of percutaneous coronary interventions or coronary artery bypass grafting [49], VSMCs undergo phenotypical switching and de-differentiation characterized by changes in proliferation, contractility and ECM production [50]. Specifically, VSMCs increase their proliferation and undergo a downregulation of contractile proteins like ACTA2, CNN1 and SM22A. Importantly, high phosphate can also promote the downregulation of contractile proteins, through its transport by the type III NaPi cotransporters (PiT-1 and PiT-2), and intracellular signaling [27]. On the other hand, as a consequence of lipid infiltration, pro-inflammatory cytokines and high phosphate VICs

proliferate. Moreover, in sharp contrast with VSMCs, VICs increase their expression of contractile proteins, resembling myofibroblasts, which express ACTA2, CNN, SM22A [51].

Nonetheless, despite this fundamental difference, VICs and VSMCs undergo similar changes in the synthesis and degradation of the extracellular matrix (ECM). Therefore, this abnormal processing of the ECM is as a key step for the thickening and calcification of valves and arteries. Two ECM molecules, widely implicated in cardiovascular calcification, and studied in the current work are elastin and type I collagen.

Type I collagen is a fibrillar protein which provides the three-dimensional framework for both mechanical strength and cellular signaling in arteries and heart valves [52]. Type I collagen is secreted by osteoblast during bone formation, serving as a scaffold for the deposition of phosphate and calcium ions [38]. In the vessels, the vast majority of the calcium phosphate mineral deposits occurs within areas of collagen fibrils, but only a small portion of the calcification occurs on the collagen fibers as in bone [53]. In the vasculature, collagen is produced by VSMCs under calcifying conditions and capable of entrapping calcifying molecules. Interestingly, in atherosclerotic plaques, mature collagen fibers surround large calcifications, whereas micro-calcifications locate in areas of low collagen density, within the gaps of the collagen fibers, rather than on the collagen. Importantly, genetic deletion of collagen degrading matrix metalloproteinase 13(MMP13) in mice, under a hypercholesterolemic background, leads to a decrease in calcification size, but not in total calcium content [54]. In line with these observations, collagen inhibits VIC calcification [55], and its disruption promotes calcification and alteration in valve phenotype [56]. This highlights the importance of collagen in the development of cardiovascular calcification.

Mechanistically, collagen does not only serve as a physicochemically favorable scaffold for the deposition of calcium and phosphate, but also as a signaling molecule. Fibrillar collagens can signal through non-integrin tyrosine kinase receptors, known as discoidin domain receptors 1 and 2 (DDR1/2). These receptors are involved in cell to collagen interactions, implicated in cell migration, proliferation, differentiation, and survival. Specifically in VSMCs, DDRs are implicated in collagen remodeling [57] and more recently DDR1 has been shown to regulate VSMC mediated calcification and fibrosis [58]. Taken together, both collagen and its sensing arise as key mechanisms in the regulation of cardiovascular calcification.

Another key ECM protein, essential for the elasticity characteristic of arteries and the pliability of valves, is elastin (ELN). Hemizygosity in the ELN gene has been associated with supravalvular aortic stenosis, characteristic of Williams-Beuren syndrome; and deletion or hemizygosity in mice leads to increase blood pressure, VSMC proliferation and tortuous arterial development [59]. *In vitro*, ELN degradation enhances calcification in VSMCs cultured with high phosphate [60]. Importantly, in nephrectomized uremic mice, ELN degradation precedes calcification [32]. In addition, elastolytic cathepsins are upregulated in AVS [61], and genetic ablation of the elastolytic enzyme cathepsin-S inhibits vascular and valvular calcification in nephrectomized mice with hypercholesterolemia [31]. Interestingly, MGP knock-out mice present disrupted ELN, and the initial calcium and phosphate deposits occur

within these ELN fibers. In addition, MGP knock-out mice, hemizygous for ELN present decreased calcification [62]. Taken together, ELN content and integrity appears essential in maintaining homeostasis of the cardiovascular system and its reduction and degradation enhances cardiovascular calcification.

1.2.2.2 Osteoblastic phenotype

Alongside the aforementioned changes in ECM and decrease in calcification inhibitors, another key event in the development of cardiovascular calcification is the osteoblastic trans-differentiation, characterized by the upregulation of calcification inducers, which leads to the organized deposition of calcium-phosphate crystals [28, 63]. Specifically, the main drivers of the osteoblastic trans-differentiation addressed in the current thesis are BMP-2 and Runx2. Among these calcification inducers one of the first to be upregulated is BMP-2 [64, 65].

BMP-2 belongs to the transforming growth factor beta (TGF-β) superfamily; essential in bone, cartilage and vascular development. BMP-2 signals through the bone morphogenetic protein receptor type 2 (BMPR2), which leads to the phosphorylation of SMAD 1/5/8 and subsequent transcriptional activation [66]. In the vasculature BMP-2 is expressed in atherosclerotic plaques [64], and its overexpression in VSMCs enhances intimal calcification, but not total atherosclerotic plaque size in $ApoE^{-/-}$ mice [67]. In line with these results, genetic [68] and pharmacological [69] inhibition of BMP-2 signaling lead to a decrease in both intimal calcification and atherosclerosis formation. In CKD patients, BMP-2 serum levels are higher than in control subjects [70]. Mechanistically, BMP-2 is upregulated by high phosphate in VSMCs. Consequentially, BMP-2 decreases SM22A, increases Runx2 expression and increases TNAP activity *in vitro* [70-72]. This suggests, that overexpression and lack of inhibition of BMP-2 correspond to the initial stages in the trans-differentiation of VMSCs and VICs leading to calcification.

Together with an increase in BMP-2 signaling, cardiovascular calcification is characterized by an increase in Runx2 expression. Runx2, alongside the transcription factor SOX9, is a master regulator of skeletal development [73]. In the cardiovascular system, nano-analytical electron microscopy has revealed that Runx2 is expressed in cells surrounding crystalline spherical particles, present in macroscopically and histologically healthy regions of human valves and vessels [53]. However, despite being present early in the calcification process, calcium-phosphate crystals and BMP-2 appear to precede Runx2 expression [65]. Nonetheless, Runx2 is essential for the calcification of the cardiovascular system. In VSMCs, Runx2 is necessary for the osteoblastic differentiation and mineralization both *in vitro* and *in vivo* [74]. In fact, targeted deletion of Runx2 in VSMCs inhibits calcification in several mouse models of calcification [74, 75]. Moreover, Runx2 has been proposed to be an inhibitor of VSMC markers, suggesting a key role in VSMC trans-differentiation and calcification [76]. Overall, Runx2 upregulation appears to be a consequence of initial calcification, but it is fundamental for the progression of calcification in the vasculature leading to the final stages in the trans-differentiation of VMSCs and VICs towards an osteoblast like phenotype.

1.2.2.3 Autophagy & Apoptosis

Together with changes in cell phenotype, hyperphosphatemia and hypercalcemia can lead to VIC and VSMC apoptosis. Specifically, calcium promotes VSMC apoptosis and vesicle release, which in turn serve as nucleating structures for calcium and phosphate deposition. A pathway through which cells protect themselves from calcification is autophagy. Autophagy is a highly conserved mechanism involved in the recycling and catabolism of proteins [77]. Recently, autophagy has been shown to be a protective mechanism, safeguarding VSMCs from phosphate induced calcification [78]. In VICs, autophagy serves as an inhibitor of osteoblastic trans-differentiation [79], as well as a survival mechanism [80] ; its inhibition leads to increased calcification in VSMCs, and decreased viability in VICs.

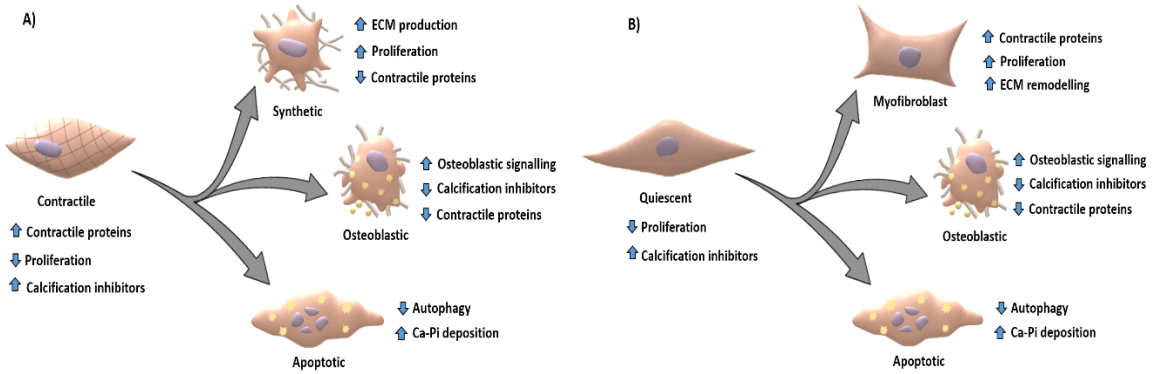

Figure 1. VSMC and VIC phenotypic trans-differentiation and cell fate: A) Under homeostasis VSMCs present a contractile phenotype. Under stress VSMCs can trans-differentiate into a synthetic or osteoblastic phenotype, as well as undergo apoptosis. **B)** Under homeostasis VICs present a quiescent phenotype. Under stress VICs can trans-differentiate into a myofibroblast or osteoblastic phenotype, as well as undergo apoptosis.

1.3 Omega-3 and Vascular Calcification

As previously alluded to, vascular calcification is preceded by apoptosis; the apoptotic cells and apoptotic bodies act as nucleating structures for calcium-phosphate crystal formation. Under physiological conditions, apoptotic cells in the vessel wall are removed by specialized macrophages, thus promoting the resolution of inflammation. However, failure in the resolution of inflammation, characteristic of atherosclerosis and aortic stenosis, favors the production of dystrophic calcification [81].

Resolution of acute inflammation is an active process mediated by a diverse set of factors including but not limited to: cytokines (IL-10), cells (regulatory T cells), and lipid mediators. The latter represent a class of bioactive lipids produced endogenously that signal through G protein coupled receptors (GPCRs) and promote the resolution of inflammation. In particular, resolvins are bioactive lipid mediators derived from the omega-3 polyunsaturated fatty acids (PUFAs): eicosapentanoic acid (EPA), and docosahexaenoic acid (DHA)[82].

Administration of EPA to rats decreases warfarin-induced calcification, as well as macrophage infiltration [83]. In addition, a recent report showed that dietary supplementation of EPA in klotho-deficient mice prevents vascular calcification without modification in

phosphate or calcium serum levels. In these mice, EPA modifies the expression and activity levels of NADPH oxidase, mediated by the receptor GPR120, however the effect of this receptor in calcification was not explored. Interestingly, the authors found increased resolvin E1 (RvE1), a lipid mediator derived from EPA, in the serum of EPA treated mice [84].

Moreover, in humans, a prospective cohort study has shown that serum levels of omega-3 PUFAs correlate with lower incidence of coronary artery calcification [85]. Taken together, these data show that omega-3 PUFAs reduce cardiovascular calcification; however, the mechanism of action remains elusive.

Some work trying to elucidate the mechanism of action of DHA and EPA has been carried out using calcifying vascular cells (CVCs), a subpopulation of bovine aortic medial cells that undergo spontaneous osteoblast differentiation and calcification. Under this experimental conditions, DHA reduces osteoblastic differentiation, and TNAP. DHA promotes the phosphorylation of p38-mitogen-activated protein kinase (MAPK), alongside the activation of the peroxisome proliferator-activated receptor-γ (PPAR- γ)[86]. These experiments shed light on the potential pathways involved in the reduction of vascular calcification by omega-3 PUFAs; however, they do not address whether these effects are mediated directly by DHA and EPA or their metabolites, or if these effects are receptor dependent or the consequence of changes in the membrane composition.

Another piece of evidence helping us understand the mechanism by which omega-3 PUFAs act, in particular resolvins, has been obtained in osteoblasts. Osteoblast cultures treated with RvE1 increased OPG production under pro-inflammatory conditions, without modifying TNAP or Runx2. In the same study, the authors generated a transgenic mouse overexpressing the receptor for RvE1, the chemokine like receptor 1 (ChemR23). Transgenic mice showed a reduction in alveolar bone loss in an experimental model of periodontitis [87], suggesting that both the ChemR23 and its ligand have a protective role in bone metabolism. However, the role of ChemR23 in vascular calcification remains unexplored.

1.3.1 ChemR23 and Bone/Cell Fate

ChemR23 is a highly conserved GPCR coupled to intracellular calcium release, cAMP inhibition, and subsequent inhibition of ERK 1/2, through Gi/o subtype G proteins. ChemR23 was originally described as the receptor for the chemoattractant protein chemerin but was later described as the receptor for RvE1[88, 89].

Chemerin is an adipokine that requires proteolytic cleavage in order to exert its functions. The main source of chemerin in the body is visceral adipose tissue. Chemerin has been associated with obesity, insulin resistance, metabolic syndrome, and CKD[90-94]. However, paradoxically, in incident dialysis patients chemerin is associated with a survival advantage[95]. These discrepancies may be attributed to the fact that chemerin can exert both pro or anti-inflammatory effects depending on the type of proteolytic cleavage carried out by proteases [96]. As a matter of fact, studies using both differentially cleaved chemerin and ChemR23 knock-out mice suggest an anti-inflammatory role [97]. Other possible explanations could

implicate the post-translational modifications of chemerin, signaling through the other chemerin receptors CCRL2 or GPR1, or ChemR23 heteromerization[98-100]. For these reasons it is important to understand which cells express ChemR23, as well as the general and cell specific functions that it exerts.

ChemR23 is expressed in a wide variety of cells including: macrophages, dendritic cells, cytotoxic natural killer cells, chondrocytes, osteoblast, endothelial cells and VSMCs. In immune cells, ChemR23 acts as a chemoattractant upon chemerin binding inducing phagocytosis under RvE1 stimulation [97].

In VSMCs, knock-down of ChemR23 decreased proliferation and migration, while stimulation with RvE1 decreases PDGF induced migration [100, 101]. In contrast, chemerin promotes proliferation and migration, while antibody neutralization of chemerin reduces carotid ligation induced intima hyperplasia [102]. This suggests that both the receptor and its ligands have an effect on VSMCs phenotype.

Interestingly, the vasculature is not the only organ where ChemR23 appears to have a key role in cell fate. In 1997, ChemR23 was found to be present during bone development [103]. More recently, the presence of ChemR23 in bone has been confirmed in mouse calvarial cultures, in tooth development, and the late stage of osteoclastogenesis[87, 104]. In osteoclasts, RvE1 has no effects on early osteoclast differentiation, but inhibits osteoclast fusion [105]. On the other hand, chemerin increases osteoclast activity *in vitro*, and this is abrogated by the ChemR23 antagonist CCX832[106]. Moreover, antibody neutralization of chemerin inhibits osteoclast differentiation and mineralized matrix resorption [107]. Taking all into account, ChemR23 appears to have a key role in cell fate, especially in bone development as well as VSMCs phenotypical differentiation.

1.4 Clinical Determination of Calcification

Multidetector computed tomography (CT) can be used to assess coronary artery calcification (CAC) and aortic valve calcification [108]. To quantify calcification different scoring methods have been developed. The standard methodology for CAC assessment is the Agaston score, calculated multiplying the calcified area, defined as an area with a peak intensity >130 Housenfield units (HU) within a 3mm slice, by a density factor. The density factor is based on the maximum HU within the area of interest [109]. Apart from the Agatston score other measurements have been developed to closer mirror the physical properties of the calcified area. Firstly, the calcium volume, multiplies the area of calcification per slice, times the slice thickness and adds the volumes of all the slices with calcification. Unlike the Agatston score, the calcium volume measurement does not take the density into account. Secondly, the calcium mass score aims to determine the actual mass of hydroxyapatite within the calcified vessel by using phantom calibration and correction factors. Finally, the calcium density score is a combination of the Agatston score and the volume score, thus providing an average density of the whole area studied [110]. CAC assessment by CT is of special importance for risk prediction of future cardiovascular events in asymptomatic individuals [111, 112]. As a matter of

fact, CAC volume is positively associated with cardiovascular disease (CVD) events, whereas CAC density shows a negative association [11], suggesting that a low CAC density might be associated with unstable atherosclerotic plaques. Interestingly, in CKD patients, where calcification is mainly present in the medial layer, both CAC score and volume linearly predict mortality. However, CAC density presents an inverse J-shaped form, meaning CKD patients within the middle tertile of CAC densities present the highest mortality [113]. Overall, this discrepancy between CKD and non-CKD patients highlights the differences between medial and intimal calcification.

Another way to measure calcification is by using positron emission tomography (PET). Unlike CT, PET provides information about the activity of a biological process. In calcification two tracers have been used: 18F-fluorodeoxyglucose (18F-FDG) and 18F-sodium fluoride (18F-NaF). 18F-FDG is used to detect inflammation around atherosclerotic micro-calcifications. This tracer is taken up by cells with high glucose consumption and the myocardium, which can obscure the assessment of calcification. On the other hand 18F-NaF preferentially adsorbs to nascent microcalcifications rather than stabilized calcification and predicts disease progression in patients with calcification of the aortic valve, making it a very attractive alternative for the early study of calcification [114, 115].

2 AIMS

The aim of this thesis is to elucidate the role of structural cell fate and ECM in the development of cardiovascular calcification. In particular, the specific aims are:

I. To determine the role of ChemR23 and its ligands on VSMCs, and in CKD-associated calcification (Articles I & II).

II. To elucidate the role of ChemR23 and omega-3 PUFAs on intimal hyperplasia and VSMC proliferation (Article III).

III. To define the role of iron in relation to AS calcification and more specifically, the effects of iron in VIC phenotype and ECM deposition (Article IV).

IV. To establish the role of first and second generation tyrosine kinase inhibitors in AVS, and their effects in VICs, in particular through interaction with collagen receptor tyrosine kinases. (Article V).

3 EXPERIMENTAL METHODOLOGY

3.1 *In Vivo* Models of Calcification

In order to study the mechanisms of vascular calcification different animal models can be used. The models of calcification can be divided into distinct groups: genetic, surgical, and pharmacological.

Genetic deletion of inhibitors of calcification causes extensive vascular calcification. MGP knock-out mice develop lethal vascular calcification and cartilaginous metaplasia at a very early stage in life [37]. At the same time deletion of eNPP1 causes vascular calcification, with normal serum and phosphate levels, as well as bone defects [116]. Surprisingly, deletion of Fetuin-A, a liver derived glycoprotein that inhibits calcium phosphate crystal growth, only causes vascular calcification in combination with other stressors such as hyperlipidemia or hyperphosphatemia [117]. Moreover, inhibition of key molecules in the homeostasis of phosphate also promote calcification. Klotho is an inhibitor of the Na/Pi co-transporter and acts as a co-receptor for fibroblast growth factor 23 (FGF23), thus promoting phosphaturia and directly inhibiting phosphate uptake by VSMCs. Klotho deficient mice develop hyperphosphatemia and, in turn, soft tissue calcification [29, 118].

Surgical intervention has also been used to model the calcification observed in CKD patients. 5/6 nephrectomy requires the surgical removal of 5/6th of the kidney, thus mimicking severe kidney disease. Within 24 weeks after 5/6 nephrectomy rats develop vascular calcification. Supplementation with high phosphorous or vitamin D3 reduces this time by 6 weeks [119]. Unlike rats, calcification in mice is only consistent in ApoE$^{-/-}$ mice on a high fat diet, 10 weeks after the nephrectomy [31, 54]. Importantly, without an ApoE$^{-/-}$ background, nephrectomy of mice does not always promote calcification, regardless of a high phosphate diet [120].

Vascular calcification can also be induced pharmacologically. An adenine rich (0.2%) diet, supplemented with phosphate (1.8%), induces mild medial calcification alongside reduced bone mineral density within two weeks, which exacerbates after 6 weeks [121]. Warfarin causes calcification of the elastic lamellae of rats within two weeks of treatment, by inhibiting the recycling of vitamin-K, hence inhibiting the carboxylation of Gla proteins[122]. Although the model is highly effective at inducing calcification in rats, mice may require longer treatment [123]. Finally, high dose vitamin D3 injections subcutaneously causes rapid calcification and bone resorption in mice. High dose vitamin D3 also induce bone resorption by increasing osteoclastic activity and inhibiting osteoblast mineralization through the vitamin D receptor, [124, 125] thus increasing calcium absorption in the intestine, decreasing calcium excretion and increasing calcium and phosphate resorption from bone. At the same time, vitamin D3 promotes intestinal phosphate absorption by increasing NaPi-IIb transporters. Apart from the systemic effects, vitamin D3 has local effects in VSMCs, and can promote VSMC osteoblastic trans-differentiation [126].

In the current thesis we used the Vitamin D3 model of calcification since it is a model which consistently induces rapid hyperphosphatemia and medial calcification in a VSMC relevant manner, without the need of complex surgical interventions or dietary supplementation.

3.2 *In Vitro* Models of Calcification

In vitro calcification is a useful tool for the high throughput pharmacological screening and for mechanistic experiments. The two most widely used methods for *in vitro* calcification are the high phosphate model and the β-glycerophosphate model.

The β-glycephosphate model is often used in combination with ascorbic acid and dexamethasone, and in occasions TGF- β. This model is based on the principle that TNAP, expressed by VSMCs and VICs, excises the glycerol moiety, thus increasing free phosphate in the cell culture media. While at the same time, ascorbic acid and dexamethasone promote collagen production [127]. With this model, VSMCs and VICs calcify after approximately 21 days. Notwithstanding that this model is useful for the study of TNAP-dependent calcification, ultrastructural analysis, of seemingly calcified nodules in porcine VICs revealed that these nodules only calcified in the outer surface, while the inner core was formed solely of collagen fibers [128].

On the other hand, the high phosphate model is based on two principles. Firstly, phosphate can act as a signaling molecule promoting the osteoblastic transformation of cells [129]. And secondly, at a certain concentration (2mM), in solution, calcium and phosphate crystals begin to precipitate within 5 days, and this precipitation is actively inhibited by cells. Moreover, only in the presence of live cells these calcium phosphate crystals are embedded in an organic amorphous matrix, resembling the structure of hydroxyapatite crystals [130]. In the current thesis the latter method was chosen because it better resembles the hyperphosphatemia in CKD patients and presents reduced calcification time.

3.3 Calcification Quantification

To quantify cardiovascular calcification several methods have been used in the current thesis. For the analysis of histological sections from either mice or humans Alizarin Red and Von Kossa stainings were used. Alizarin Red is an anthroquinone dye which preferentially stains calcium ions, resulting in a red color. Complementarily, Von Kossa is a silver nitrate based method, where silver replaces calcium present in calcium salts, resulting in black deposits. The Von Kossa staining is normally not used alone in the determination of calcium deposits in the aortic valve, as the black stain can be easily confused with the dark pigmentation of melanocytes [131].

To quantify calcification in a semi-quantitative manner several methods have been used. *In vitro* Alizarin Red staining was quantified by spectrophotometry following the extraction of the red dye with acetic acid [132]. In the later studies the OsteoImage assay was used. This method specifically labels hydroxyapatite fluorescently, thus increasing the specificity and sensitivity in the determination of calcification. This assay is a good complement to the

Alizarin Red method, since not all the calcification formed *in vitro* is hydroxyapatite. Finally, the o-cresolphthalein complexone method is another detection method for calcium ions. This method requires the dissolution of calcium salts with a strong acid, giving rise to a purple color that can be quantified by spectrophotometry. One disadvantage of this method is that it quantifies total calcium, including intracellular calcium. However, this method is compatible with protein determinations, thus allowing the normalization of calcium to total protein, making it very useful for the determination of calcification in tissues *ex vivo*.

3.4 *In Vivo* & *In Vitro* Models of Proliferation

In order to assess proliferation of VSMCs and VICs different models have been used in the current thesis. *In vitro*, thymidine incorporation and WST-1 were used to assess proliferation. On the one hand, thymidine incorporation, quantifies the incorporation of tritiated thymidine (^{3}H-Thy) in newly synthesized DNA. Making it useful for the quantification of replicating cells. On the other hand, WST-1 is a tetrazolium salt that becomes cleaved by metabolically active cells, giving rise to formazan, a soluble molecule that can be quantified by spectroscopy. In this case, WST-1 serves as a marker of cell viability, and acts as a surrogate marker of cell proliferation.

In vivo the murine carotid ligation model was used. In this model the left common carotid artery is ligated (Article III, Figure 1A), giving rise to a thrombus and thus inducing macrophage and neutrophil infiltration, as well as VSMC proliferation under inflammatory conditions[133].

4 RESULTS & DISCUSSION

The current thesis comprises of five different articles, in which the role of VSMCs, VICs and the ECM in cardiovascular calcification have been studied.

In this section, the most relevant findings of these five articles are summarized and discussed. This discussion will serve as a complement to the discussion present in the articles, as well as to contextualize them with each other.

Article I aimed to establish the role of ChemR23 in VSMC fate and calcification. Exploratory gene expression analyses in epigastric arteries from CKD patients identified COL1A1 as an independent predictor of ChemR23 gene expression. Causality behind this correlation was confirmed analyzing Col1A1 gene and protein expression in murine VSMCs lacking ChemR23. Further comparison between wild-type ($ChemR23^{+/+}$) and knock-out ($ChemR23^{-/-}$) VSMCs revealed that $ChemR23^{+/+}$ VSMCs proliferated more and were more prone to loss of contractile proteins *in vitro* than $ChemR23^{-/-}$. This difference in VSMC phenotype was accompanied by increased expression of the calcification inhibitor OPG in $ChemR23^{-/-}$ VSMCs, which is in line with our results in macrophages [134]. Nonetheless, this difference in OPG production did not appear to be the sole reason for the reduced calcification observed in $ChemR23^{-/-}$ VSMCs (Article I, Figure 3 A), as antibody adsorption of OPG did not increase $ChemR23^{-/-}$ VSMC calcification to the same degree as in $ChemR23^{+/+}$ VSMCs (Article I, Figure 3 D). Another potential mechanism by which $ChemR23^{-/-}$ VSMCs were protected from phosphate-induced calcification is the reduced osteoblastic signaling observed in $ChemR23^{-/-}$ VSMCs. $ChemR23^{-/-}$ VSMCs presented reduced Runx2 expression levels, and more importantly, they were resistant to BMP-2 induced SMAD 1/5/8 phosphorylation. This decrease in SMAD signaling could explain the lower levels of Runx2, as BMP-2 can promote Runx2 expression [70]. Interestingly, this difference in SMAD phosphorylation was independent of BMP2R or inhibitory SMADs expression (Article I, Supplementary Figure 1), suggesting a more upstream BMP-2 inhibition, such as increased MGP production. These differences are particularly interesting in light of the results from Tang et al, which show increased BMP-2 expression in $ChemR23^{-/-}$ ovaries as compared with $ChemR23^{+/+}$, and propose ChemR23 signaling to increase BMP-4 expression [135], although the mechanism remains elusive. Nonetheless, these results suggest a link between ChemR23 signaling and BMPs.

It is worth noting that the differences observed *in vitro*, specifically on VSMC phenotype, ECM production and calcification, could be reproduced *in vivo* in a vitamin D3-induced mouse model of medial calcification. Interestingly, we also observed reduced bone mineral density in $ChemR23^{-/-}$ mice, suggesting that ChemR23 plays a role in the preservation of bone. As a matter of fact, ChemR23 deficiency has been linked to reduced testosterone levels in male mice, consequently leading to reduced bone mineral density [136]. Moreover, in MSCs, ChemR23 deficiency favors adipogenesis over osteoblastogenesis [137], which to some extent, could explain the difference observed in vascular calcification in this study and the increase

in atherosclerosis that we reported in ChemR23$^{-/-}$/ApoE$^{-/-}$ mice [134]. Finally, in **Article I**, the ChemR23 ligand, RvE1, could inhibit both VSMC calcification and BMP-2 gene expression through ChemR23 *in vitro*, without altering VSMC phenotype. *In vivo*, by introducing the Fat-1 transgene, we could show that endogenous production of omega-3 PUFAs resulted in a reduction in the inhibition of calcification as a result of ChemR23 deletion (Article I, Supplementary figure 2). Overall, these experiments suggest that the previously observed effects of omega-3 PUFAs on vascular calcification, could in part be mediated through the inhibitory signaling of ChemR23 by RvE1.

As mentioned previously, chemerin is the other ligand of ChemR23. However, chemerin has been associated with an increased cardiovascular risk [90] and with CKD [93]. Paradoxically, despite this dismal associations, chemerin is associated with a survival advantage in incident dialysis patients [95] suggesting that these increases in chemerin, at least in CKD patients, might be protective. Therefore, based on our results from **Article I** on the role of ChemR23 signaling and calcification, we hypothesized that chemerin was exerting its protective effects in CKD patients by inhibiting calcification through ChemR23. For that reason, and to further elucidate the role of the ChemR23 pathway in vascular calcification, in **Article II** we aimed to establish the predictive value of chemerin as a biomarker for coronary calcification and determine the effects of chemerin signaling, through ChemR23 in VSMC calcification.

In **Article II**, we confirmed that CKD patients present higher levels of chemerin than healthy controls. Importantly, we showed that chemerin was inversely associated with CAC score, even after adjustment for confounders (Article II, Figure 1B). Moreover, we observed that chemerin was positively associated with circulating levels of Fetuin A and MGP. To assess the causality behind these associations we treated murine VSMCs with chemerin under calcifying conditions, observing a reduction in calcification in the chemerin treated cells. Mechanistically, we could show that chemerin reduced calcification and increased MGP expression through ChemR23. Overall, chemerin arises as a potential inhibitor of vascular calcification, which acts locally in VSMCs, directly inhibiting calcification and increasing MGP; and can act systemically increasing fetuin A.

In summary, in **Article I** and **Article II**, ChemR23 arises as a determinant of VSMC synthetic phenotype, with increased proliferation, collagen production, and susceptibility to phosphate-induced osteoblastic trans-differentiation and medial calcification. This increase in calcification could be inhibited with the ChemR23 ligands RvE1 and chemerin. Importantly, this pathway appeared relevant for CKD, as chemerin was inversely associated with CAC score in a cohort of CKD patients.

So far **Article I** and **Article II** have explored the role of ChemR23 in VSMCs under non-inflammatory conditions. However, work from our laboratory has demonstrated that under inflammation and hypercholesterolemia, characteristic of ApoE$^{-/-}$ mice, ChemR23 deletion increases atherosclerotic plaque size [134] and consequently intimal calcification (Figure 2). This effect in atherosclerosis was mediated by macrophages which present increased oxidized low density lipoprotein (oxLDL) uptake and reduced phagocytosis. This difference in

macrophage phenotype could also explain why the atherosclerotic plaques were more calcified, especially, since macrophages stimulated with oxLDL express higher TNAP [138] and M2 macrophages inhibit VSMC calcification *in vitro* by increasing extracellular Ppi [139]. Moreover, macrophages can phagocyte hydroxyapatite particles [140, 141], therefore a decrease in phagocytosis could potentially translate into a decrease in the clearance of calcium-phosphate crystals. Another explanation for this difference in atherosclerosis and intimal calcification may depend on changes in the VSMC phenotype under inflammatory conditions. To test the latter hypothesis, in **Article III**, we used the carotid ligation model to induce intimal hyperplasia.

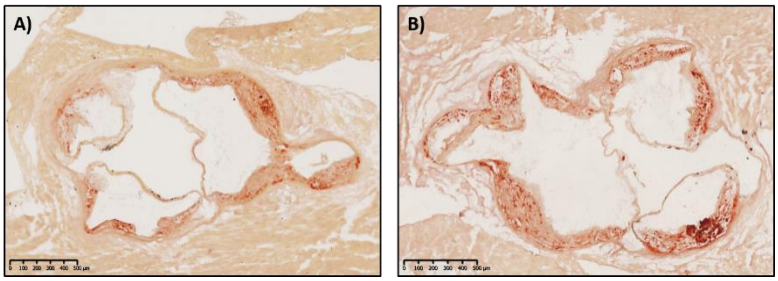

Figure 2. Intimal calcification in ApoE$^{-/-}$ mice: Representative photomicrographs of Alizarin Red stained aortic roots from:
A) ApoE$^{-/-}$/ChemR23$^{+/+}$
B) ApoE$^{-/-}$/ChemR23$^{-/-}$

The aims of **Article III** were to establish the role of ChemR23 in VSMC proliferation under inflammation, and determine the downstream signaling of omega-3 PUFAs in intimal hyperplasia. Analysis of intimal hyperplasia after carotid ligation revealed that ChemR23 deletion significantly increased intimal hyperplasia. Interestingly, increasing omega-3 production by means of Fat-1 transgene led to a reduction of intimal hyperplasia in both ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice (Article III, Figure 1B). This finding was surprising in light of another report showing that RvE1 supplementation inhibited wire-injury induced VSMC migration [142]. However, the direct involvement of ChemR23 *in vivo* was not studied. Overall, our results suggest that in our model: RvE1 may signal through its other receptor, BLT1; that other lipid mediators, such as RvD1, could promote the inhibition of intimal hyperplasia [143]; or that the beneficial effects of omega-3 PUFAs in intimal hyperplasia could be mediated through the free fatty acid receptor-4 [144].

At the same time, the genotype effect observed between ChemR23$^{+/+}$ and ChemR23$^{-/-}$ in **Article III**, was in sharp contrast with the effects on proliferation observed in **Article I**, those reported by Liu et al silencing ChemR23 in VSMCs [100], and the reduction in intimal hyperplasia reported by Xiong et al after silencing chemerin [145]. To clarify this dichotomy, we hypothesized that the differential effects observed between ChemR23$^{+/+}$ and ChemR23$^{-/-}$ mice, after carotid ligation, were caused by differences in inflammation. Immunohistochemical analysis of total neutrophil and macrophage content revealed no significant differences between the genotypes (Article III, Supplementary Figure 1). However, in support of previous reports [146] [147], gene expression analyses showed that ChemR23$^{-/-}$ macrophages were more pro-inflammatory. Functionally, this difference between ChemR23$^{+/+}$ and ChemR23$^{-/-}$ macrophages resulted in VSMCs proliferating more when cultured with conditioned media derived from ChemR23$^{-/-}$ macrophages. These experiments revealed that the same receptor can exert both protective and deleterious effects depending on

the degree of inflammation. In line with these results, chemerin can also exert pro or anti-inflammatory effects depending on the type of proteolytic cleavage carried out by proteases, and secreted by macrophages [96]. This differential, chemerin cleavage, could explain why some reports have observed increased proliferation with chemerin [102], and others a decrease [148].

In summary, **Articles I**, **II** and **III** demonstrate that ChemR23 acts as a dual-role receptor dependent on the cell type in which it is expressed, as well as the degree of inflammation. In VSMCs, in the absence of inflammation, ChemR23 promotes proliferation, ECM production, and phosphate-induced calcification. Moreover, ChemR23 deletion can revert this trans-differentiation of VSMCs, and, akin to RvE1 and chemerin, inhibit phosphate-induced calcification. However, in macrophages, ChemR23 promotes the resolution of inflammation, and its deletion enhances atherosclerosis, as well as intimal hyperplasia. In conclusion, these articles suggest that while ChemR23 promotes the resolution of inflammation in macrophages, ChemR23 may transduce deleterious effects on VSMCs under non-inflammatory conditions.

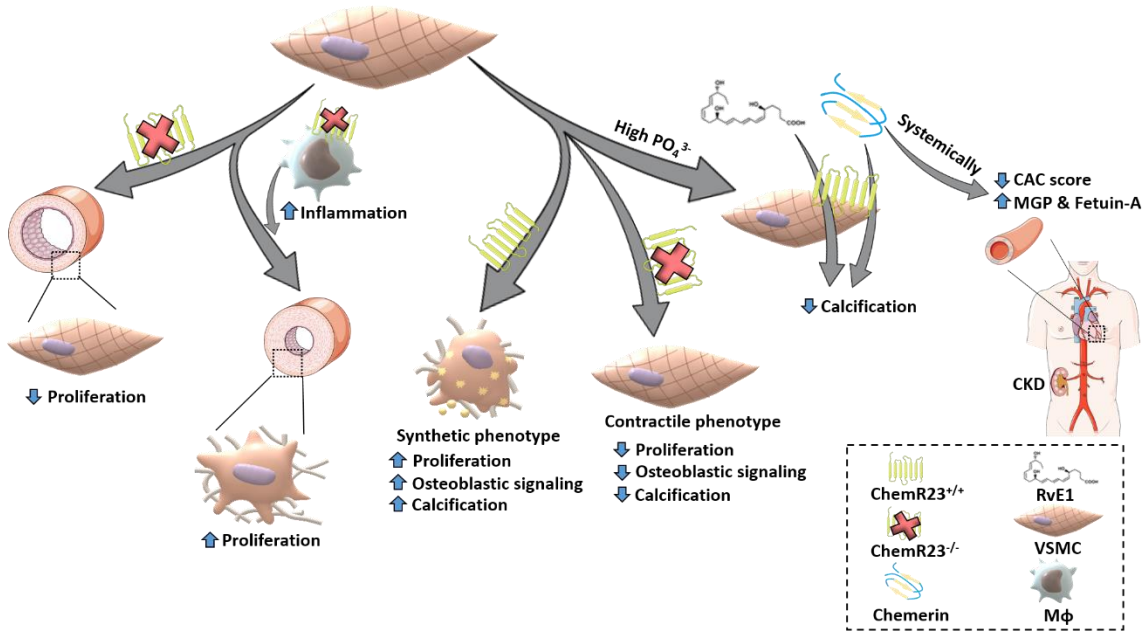

Figure 3. Schematic summary of articles I, II & III: VSMC phenotype is altered by ChemR23. ChemR23 promotes a synthetic phenotype prone to phosphate-induced calcification and osteoblastic signaling, this phenotype is reversed by the genetic deletion of ChemR23. The two ChemR23 ligands: RvE1 and chemerin decrease phosphate-induced calcification through ChemR23. Moreover, in a cohort of CKD patients, chemerin is associated with a reduced CAC score and an increase in the calcification inhibitors MGP and Fetuin-A. Finally, ChemR23 deletion enhances intimal hyperplasia and therefore VSMC proliferation through more inflammatory macrophages.

In **Article IV**, we hypothesized that the non-heme bound iron derived from extravasated red blood cells (RBC), from intraleaflet haemorrhages, initially described by Akahori et al [25], could be a driver of the progression of AVS. Based on this hypothesis, we aimed to establish the role of iron in relation to AVS calcification; more specifically, the effects of iron in VIC phenotype and ECM deposition.

Stratification of 36 valves, from AVS patients, based on their iron positivity, measured by Pearl´s blue staining, revealed a positive association with the degree of calcification. In addition, iron positivity correlated with MMP9 expression, suggesting an effect of iron on ECM degradation. As mentioned in the introduction, VICs are the most abundant cell type of the valve and their phenotypic transformation is considered a key driver in the progression of AVS. Two key cytokines involved in this process are tumor necrosis factor alfa (TNF-α), and TGF-β [149]. Stimulation of VICs with these cytokines resulted in a decrease in expression of the iron exporter ferroportin (FPN1), while TNF-α stimulation also increased the expression of the divalent metal transporter (DMT1), an importer of iron. Suggesting, that VICs could uptake iron, and that relevant pathophysiological pathways can favor this uptake. In support of these results, VICs from control patients expressed significantly higher levels of FPN1.

To understand if VICs were causally involved in the retention of iron in the calcified valves, we treated VICs with iron sulfate. These experiments revealed that VICs could, in a time-dependent manner, take up iron. Finally, to determine whether iron can alter VIC phenotype, and thus contribute to the disease progression, we analyzed the effect of iron on VIC proliferation and elastin deposition. Iron significantly increased VIC proliferation. This switch from a quiescent state towards a proliferative one is one of the earlier stages in the disease [51], suggesting that iron could contribute to an increase in valve thickening. These results are in line with those observed in human hepatic carcinoma cells where iron promotes proliferation and chemerin inhibits this effect by downregulating DMT1 and transferrin receptors, leading to cell cycle arrest [148]. In addition, in **Article IV**, iron significantly decreased elastin production. This decrease in elastin, accompanied by the observed increase in MMP9 in valves and our recently reported increase in cathepsin expression in the calcified tissue of human valves [80], support the role of iron as a potential modifier of the ECM in the valve, that can contribute to the progression of AVS. Importantly, building up on these results, a recent report has shown that early iron deposits are present in seemingly healthy areas, without alizarin red detectable calcification, of human aortic valves. The authors suggest that this iron comes from RBCs that permeate through the damaged endothelium; and that senescent RBCs can induce VIC osteoblastic signaling [24].

In conclusion, **Article IV** demonstrates that iron accumulates in the VICs of the aortic valve, actively contributing to the ECM remodeling, VIC proliferation, and potentially osteoblastic trans-differentiation.

In addition to elastin, another key structural component of the aortic valve is collagen. Recently, disrupted collagen sensing and content have risen as a potential mechanism in the development of vascular and valvular calcification, respectively [56, 58]. Importantly, the

collagen sensing tyrosine kinase receptors DDR1 and 2 can be inhibited by the tyrosine kinase inhibitors (TKIs), clinically used in the treatment of chronic myeloid leukemia (CML). In **Article V**, based on the clinical observation of a young woman who developed rapid aortic stenosis progression upon initiation of nilotinib for the treatment of CML, we hypothesized that the second generation TKI nilotinib exerts detrimental effects in the valve through the inhibition of DDRs. Therefore, in **Article V** we aimed to establish the role of first and second generation TKIs in AVS, and determine the associated molecular mechanisms. Treatment of APOE*3Leiden.CETP transgenic mice with the first (imatinib) and second (nilotinib) generation TKIs revealed that nilotinib treated mice presented thicker valves than those treated with imatinib. An increase in valve thickness is one of the hallmarks in the initiation of AVS, and is associated with increased proliferation, ECM remodeling, and calcification. To discover the pathways behind nilotinib's increase in valve thickness, we explored the gene expression levels of the tyrosine kinases (TKs), targeted by nilotinib, in the healthy and calcified tissue of human aortic valves. Among all the TKs explored, only DDR2 was both among the highest expressed in healthy tissue and the most downregulated in calcified tissue. Importantly, in patients with renal disease, DDR2 was already downregulated in the thickened tissue. To determine if hyperphosphatemia (one of the key hallmarks of CKD) was involved in this premature downregulation of DDR2 we cultured VICs under calcifying conditions induced by high phosphate. This experiment revealed that phosphate reduced DDR2 expression in VICs, suggesting a direct role of phosphate in DDR2 downregulation. Further analyses in VICs, revealed that DDR2 was preferentially expressed over DDR1 and that only nilotinib inhibited both DDR1 and 2, whereas imatinib only inhibited DDR1. Importantly, in lung fibroblasts, Col1A1 signaling is dependent exclusively on DDR2 activity [150], suggesting that this differential regulation of DDRs might be particularly important in the interplay between VICs and the ECM. Moreover, treatment with nilotinib enhanced VIC calcification and promoted osteogenic signaling in VICs by increasing Runx2 and BMP-2 expression. Finally, nilotinib significantly inhibited VIC autophagic flux.

Overall, in **Article V**, our results show that *in vivo* nilotinib promotes aortic valve thickening, and *in vitro* nilotinib promotes VIC osteoblastic trans-differentiation and calcification. At the same time, nilotinib inhibits autophagy, a pathway involved in VIC osteoblastic trans-differentiation, and survival. Moreover, we show that pathways relevant to collagen sensing are altered in AVS, with a preference for DDR2. This difference in DDR expression might be of particular relevance for the field of cardio-oncology, in light of the differential inhibition that first and second generation TKIs present for these receptors.

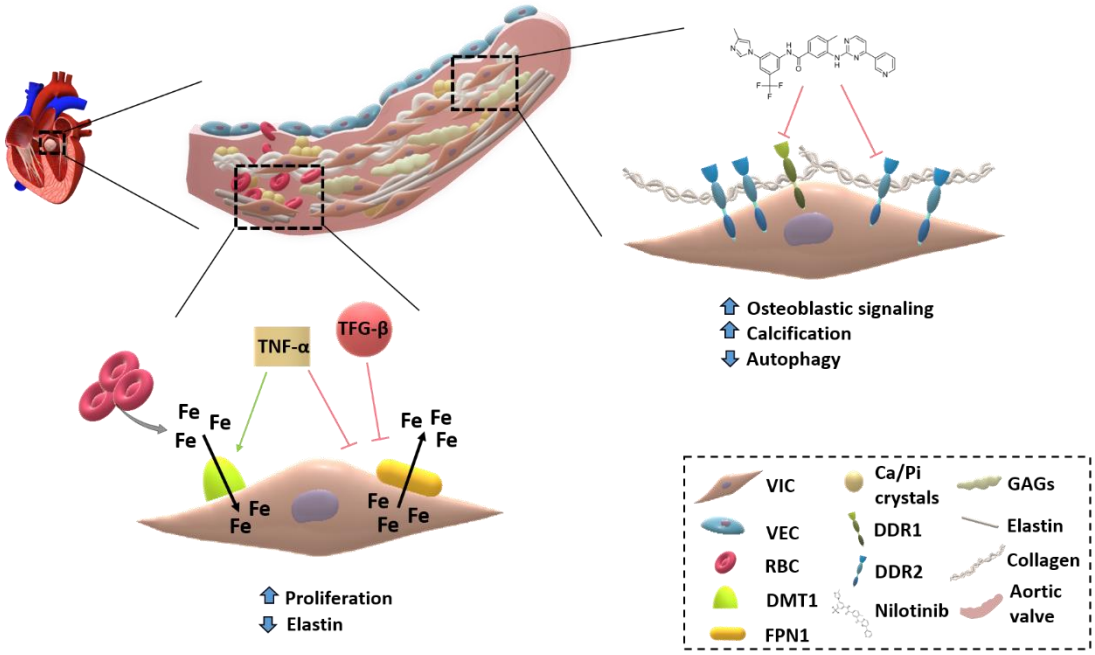

Figure 4. Schematic summary of articles IV & V: Iron from extravasated RBCs is taken up by VICs through DMT1, promoting VIC proliferation and a decrease in elastin production. The expression of the iron importer DMT1 is induced by TNF-α, whereas the expression of the iron exporter FPN1 is inhibited by both TNF-α and TGF-β. DDR2 is preferentially expressed over DDR1 in human aortic valves. The nilotinib inhibits both DDR1 and 2 promoting an increase in the osteoblastic signaling, calcification and a decrease in autophagy.

5 SUMMARY & CONCLUSIONS

In summary, four key findings arise from the current thesis: First, the GPCR, ChemR23 has profound effects in VSMC phenotype and ECM production, promoting a synthetic and proliferative phenotype, which translates into an increase in medial calcification under hyperphosphatemia. Importantly, this increase in calcification can be inhibited with the ChemR23 ligands RvE1 and chemerin. Secondly, under inflammation, ChemR23, expressed in macrophages, promotes the resolution of inflammation, thus inhibiting VSMC proliferation. Third, inorganic ions like iron, and not just phosphate and calcium, can modify the phenotype of VICs leading to ECM remodeling and consequently valvular calcification. Finally, inhibition of the ECM sensing, by the TKI nilotinib, can promote valve thickening, VIC osteoblastic trans-differentiation and calcification.

In conclusion, the ChemR23 pathway arises as a key determinant of a synthetic VSMC phenotype, contributing to vascular calcification. Importantly, both ChemR23 ligands, RvE1 and chemerin, inhibited calcification through ChemR23, thus revealing ChemR23 as a pharmacologically relevant pathway for cardiovascular calcification. At the same time, in the aortic valve, alterations in the ECM appear as both a cause and a consequence in the phenotypic trans-differentiation of VICs leading to AVS.

6 ACKNOWLEDGEMENTS

"I have passed through fire and deep water, since we parted. I have forgotten much that I thought I knew, and learned again much that I had forgotten." Gandalf.

Acknowledging those who have helped me or inspired me along this journey is a tough but exciting task. Like every journey it has a beginning and, for now, an end.

For that reason, I shall begin acknowledging my family. My parents, **Marta Ortiz** and **José Carracedo**, who have always professed me unconditional love and granted me innumerable opportunities, regardless of cost or complications. My brother, **Alvaro Carracedo**, who always has my back, independently of how many of his Legos I decapitated as a child. My uncle, **Alberto Ortiz**, who has always inspired my competitive side. My grandmother, **África**, who has always nurtured my curiosity with books. And my other grandmother, **Angelita**, from whom I learned to be skeptical.

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The next stage in this trip takes place almost six years ago in Stockholm. During this time, I have developed as a scientist, a runner and a beer connoisseur. And this has in turn meant meeting countless people.

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